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# Tetrapeptide Inhibitors of the Glutamate Vesicular Transporter (VGLUT)

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## Abstract

Quinoline-2,4-dicaboxylic acids (QDCs) bearing lipophilic substituents in the 6- or 7-position were shown to be inhibitors of the glutamate vesicular transporter (VGLUT). Using the arrangement of the QDC lipophilic substituents as a template, libraries of $X_1X_2EF$ and $X_1X_2EW$ tetrapeptides were synthesized and tested as VGLUT inhibitors. The peptides QIEW and WNEF were found to be the most potent. Further stereochemical deconvolution of these two peptides showed $_{D}Q_{L}I_{D}E_{L}W$ to be the best inhibitor ($K_i$ = 828 ± 252 μM). Modeling and overlay of the tetrapeptide inhibitors with the existing pharmacophore showed that H-bonding and lipophilic residues are important for VGLUT binding.

L-Glutamate is stored in synaptic vesicles prior to its depolarization-triggered, calcium-dependent release from neuron terminals[1-4] and is transported into the vesicles in an ATP-dependent manner by the glutamate vesicular transporter (VGLUT). Unlike the plasma membrane neurotransmitter transporters, VGLUT is stimulated by low, physiologically relevant concentrations of $Cl^-$ ion,[5] although the contribution of the $Cl^-$ - to ΔpH has been debated.[2-7] VGLUT is specific for glutamate but it is low affinity ($K_m$ = 1 to 3 mM), which contrasts with the plasma membrane transporters that are specific for glutamate but high affinity ($K_m$ = 5-50 μM).[8-11] To differentiate between these transporters, potent and selective inhibitors of VGLUT are needed.

The main VGLUT inhibitor structures have been recently reviewed.[1] In brief, aspartate[5,12] and simple glutamate analogs are not good inhibitors of VGLUT, whereas some kynurenate analogs showed modest activity. The alkaloid bromocryptine ($k_i$ = 20 μM) and certain azo dyes (e.g., trypan blue) are among the most potent VGLUT inhibitors (Fig. 1).[13] We reported a systematic, structure-activity study of quinoline 2,4-dicarboxylic acids (QDCs; Fig . 2) as inhibitors[14, 15] that seeded the development of the first pharmacophore model[1] for VGLUT and the use of QDC's as a key motif for future inhibitor design and substituent variation.

Some of the more potent QDC-based inhibitors contained lipophilic groups at position 6 or a hydroxyl at position 8. Combining these favorable substituents into the QDC template led to the observation that this pattern overlays with a peptide that contains ($HO_2C$)WEX($NH_2$) (Fig. 2). The very weak basicity of the QDC nitrogen also suggested that a peptide amide might be an appropriate isostere. This prompted an investigation of small peptides that might be capable of binding VGLUT. Peptide-based inhibitors are also possible leads to uncover protein interactions.

Based on observations that QDCs containing an embedded glutamate moiety and lipophilic substituents (phenyl, styryl, etc.) confer greater inhibitory activity, a tetrapeptide library was envisioned in which the C-terminus amino acid was occupied by either tryptophan (W) or phenylalanine (F) to represent the lipophilic substituent and the adjacent position occupied by a glutamate (E) residue. The N-terminus and second residue ($X^1$ and $X^2$) were systematically varied to investigate how these positions could enhance binding (Fig. 3).

To further refine our binding requirements and increase the overall library diversity, either D- or L-amino acids were used. Further rationale for the incorporation of D-glutamate into the libraries is based on the modest activity of this enantiomer as an inhibitor of VGLUT.[11] Overall, stereoisomeric tetrapeptides $X^1X^2$EW(F) were prepared and evaluated as VGLUT inhibitors (Fig. 3; Table 1).

*Peptide Synthesis*. [16] Tetrapeptides were synthesized according to Scheme 1 and structures determined by NMR and/or mass spectrometry.

*Inhibition of VGLUT by Tetrapeptides*. [17] Screening of the peptide libraries as VGLUT inhibitors was carried out using $^3$H-glutamate as substrate and the ability of test compounds to block the uptake of $^3$H-glutamate into synaptic vesicles isolated from rat forebrain. Tetrapeptide sub-libraries of the type $X^1X^2$EW (Library 1) or $X^1X^2$EF (Library 2), where $X^1$ and $X^2$ were varied as amino acids (AA) in D- or L- form (except cysteine), and where the identity of $X^1$ was known, were tested as inhibitors of VGLUT.[18] The sub-libraries were screened and the pools showing the most inhibition of uptake were deconvoluted to identify $X^2$ residues (Table 1).

We next reasoned that the various stereoisomers comprising a deconvoluted sublibrary would be approximately equal, allowing the assay to be a reasonable guide to selection of the pools used for further deconvolution. In library 1, the only tetrapeptide sublibrary to inhibit glutamate uptake at VGLUT were structures with $X^1$ = Q (56% uptake). Systematic screening of the Q$X^2$EW tetrapeptides indicated the order of inhibitory potency for position $X^2$ as I ≫ Q, W> N. Therefore, QIEW was selected for deconvolution into its stereoisomers, six of which inhibited the uptake of glutamate: LDLD (28%), DDLD (35%), LDDD (60%), DDDD (63%), LDLL (69%), and LLDL (82%). The QWEW panel was active but allowed 66% residual transport. Based on the lesser activity and solubility limits, this panel was not deconvoluted for further analysis.

The results from Library 2 showed that aryl or amide residues are preferred at position $X^1$ (N-term) with less uptake allowed following the order W (36%) < N (63%). As found with Library 1, Library 2 showed activity with a wider range of possible residues occupying position 2. For $X^2$ in Library 2 (W$X^2$EF), the order was N (13%) > Q, W > I, F > H. As with library 1, the WNEF tetrapeptide was selected for further stereoisomer deconvolution. Interestingly, the individual enantiomers were not as active as the mixture and only one isomer, DWLNDEDF showed inhibition of glutamate uptake (41%).

To better understand the inhibitory activity, a more thorough analysis of DQLIDELW was conducted. Using the Cheng-Prusoff relationship to estimate from $IC_{50}$ values, the $K_i$ was determined from non-linear regression analyses of sigmodial inhibitory dose-response curves. The inhibitory dose-response for DQLIDELW was plotted (Fig. 4) and the $K_i$ was determined to be 828 ± 252 μM as the mean ± s.e.m (n = 3). In separate experiments, it was determined that neither QIEW nor WNEF blocks v-ATPase activity in rat synaptic vesicles at the concentrations tested for VGLUT inhibition.

Although a glutamic acid is fixed at position 3 in all libraries, charged residues at positions $X^1$ and $X^2$ resulted in poor VGLUT inhibition. For $X^2$, both libraries prefer hydrophobic or amide-containing residues. Q, N, W, and I consistently appeared in the panels that showed

inhibition of glutamate uptake. This finding both correlates with the structure of bromocryptine (Fig. 1),[19] a 'peptide containing' alkaloid and with the reportedpharmacophore. Bromocryptine is a potent inhibitor that lacks an acidic side chain group like glutamate but contains homologous lipophilic residues along a peptide-like framework.

In an effort to gain insight into the plausible interactions with VGLUT as a function of the QDC-based ligand design features (Fig. 2) and the inhibition (Table 1, Fig. 3) an initial alignment of an extended conformation of the stereoisomer $_{L}Q_{D}I_{L}E_{D}W$ (C- to N-terminal) was made against our established inhibitor-based VGLUT pharmacophore model (Fig. 5).[1]

The inhibitor pharmacophore model, derived using the automated three-dimensional (3D) ligand alignment protocol GASP (Tripos Inc., St. Louis), is defined with 6-(4'-biphenyl)-quinoline-2,4-dicarboxylate (QDC, grey), one half of the symmetric structure of Chicago Sky Blue (yellow, CSB) and bromocryptine (orange, BCP). The superposition model describes three distinct regions in 3D space, including γ-carboxyl groups, H-bonding acceptors and lipophilic pocket moieties brought together with discrete inter-atomic distances between the regional points (Å values, Fig. 5). Since small peptides may assume a host of conformations, a plausible extended conformation of $_{L}Q_{D}I_{L}E_{D}W$ was selected for a VGLUT pharmacophore model comparison.

Analysis of the $_{L}Q_{D}I_{L}E_{D}W$ conformation alignment reveals that the N-terminal arginine (Q) correlates to the γ-carboxyl model region, the glutamic acid (E) side chain carboxyl moiety corresponds to the pharmacophore H-bonding acceptor group, and the C-terminal tryptophan (W) is consistent with the aromatic ring lipophilic pocket superposition area. Taken together, QIEW may block glutamate transport at VGLUT because it contains three ligand structural binding elements similar in 3D space to the more potent inhibitor ligand pharmacophore groups. Although the lesser potency of QIEW likely results from the conformational flexibility, its identity as a VGLUT inhibitor is consistent with the basic QDC-template peptide design strategy (Figure 2).

Moreover, examination of the Q-$X^2$-E-W library reveals an enhanced peptide inhibition efficacy when $X^2$ = isoleucine (I). Thus, ligands with terminal aromatic ring groups and an additional lipophilic side chain residue (e.g., W and I of QIEW; W and F of WNEF, not shown) might be related to the distinct dual lipophilic moieties (the terminal aromatic rings and distal propyl groups) of the more potent VGLUT inhibitor bromocryptine. Collectively, the lipophilic peptide and bromocryptine moieties could be important structural facets for enhanced VGLUT inhibition. Full conformational analyses of QIEW and WNEF and their superposition within the pharmacophore model are currently underway to discern the relative 3D arrangement of the lipophilic groups.

The discovery of tetrapeptide inhibitors raises the possibility that they may represent protein motifs that may bind VGLUT. BLAST analysis (*rodentia*)[20,21] matched a $Ca^{+}$-transporting ATPase (WNEF), neuroprotective protein (QIEW), and vasohibin (QIEW). We are currently examining the possibility of protein binding to VGLUT using these leads.

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16. Peptides were synthesized with DIPCDI/HOBt using Fmoc methodology on a Cl-Trt-Cl (2-chlorotrityl chloride) resin and cleaved from the resin bead by treatment with 4:1 $CH_2Cl_2$/HOAc for 3 h. Peptides were treated with TFA to remove protecting groups.
17. Vesicular transport was quantified as described Kish PE, Ueda T. Methods Enzymol 1989;174:9. [PubMed: 2517314]Assays were initiated by the addition of $^3$H-L-glutamate ± inhibitors (0.01-5 mM) to the synaptic vesicles (approx. 0.1 mg protein). Rates of uptake were normalized to protein content
18. Highly lipophilic peptides displayed poor solubility and were first solubilized in acetonitrile and then diluted into the HEPES assay buffer.
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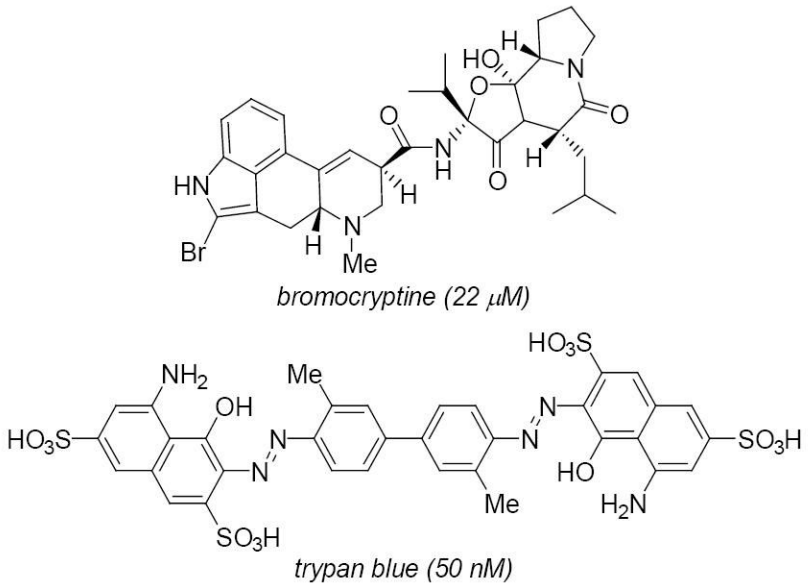



**Fig 1.**
Structures of VGLUT inhibitors

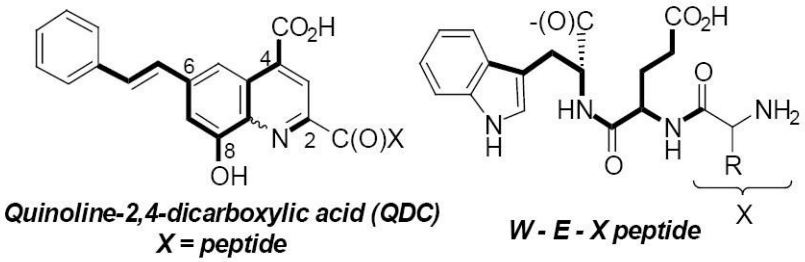



**Fig 2.**
Proposed QDC inhibitor structural relationship to peptides.

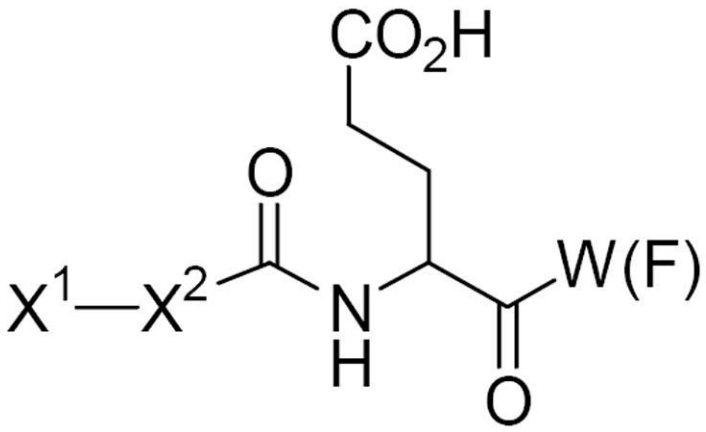



**Fig 3.**
Tetrapeptide design based on the QDC-template.

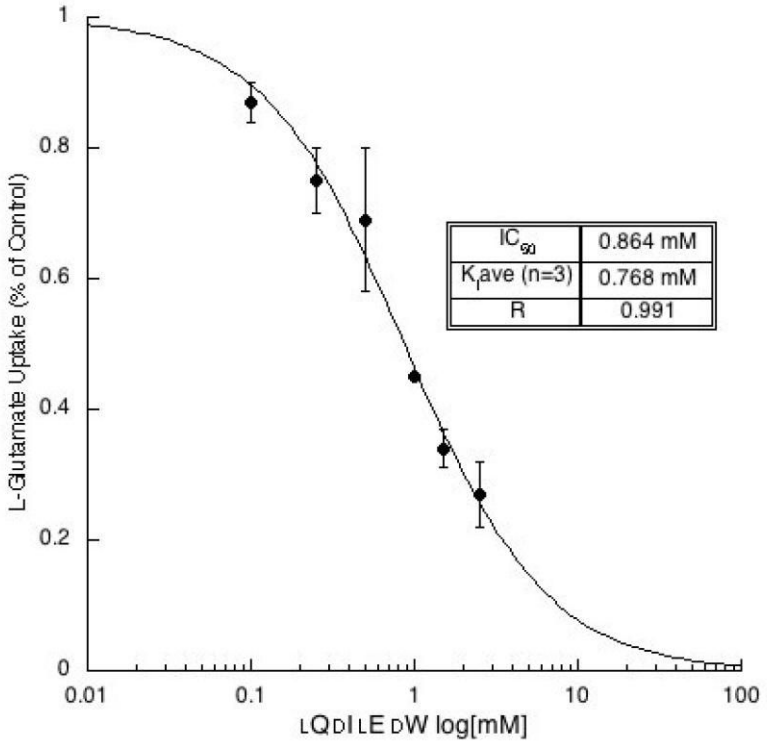



**Fig 4.**

Demonstration of the inhibitory dose-response dependency of $_{D}Q_{L}I_{D}E_{L}W$ on the uptake of $^{3}H$-$_{L}$-glutamate (0.25 mM) into rat brain synaptic vesicles. Representative plot yielded a $K_i$ = 0.465 mM. The control rate for L-glutamate uptake was 1912 nmol/min/mg protein and $K_m$ = 2.1 mM.

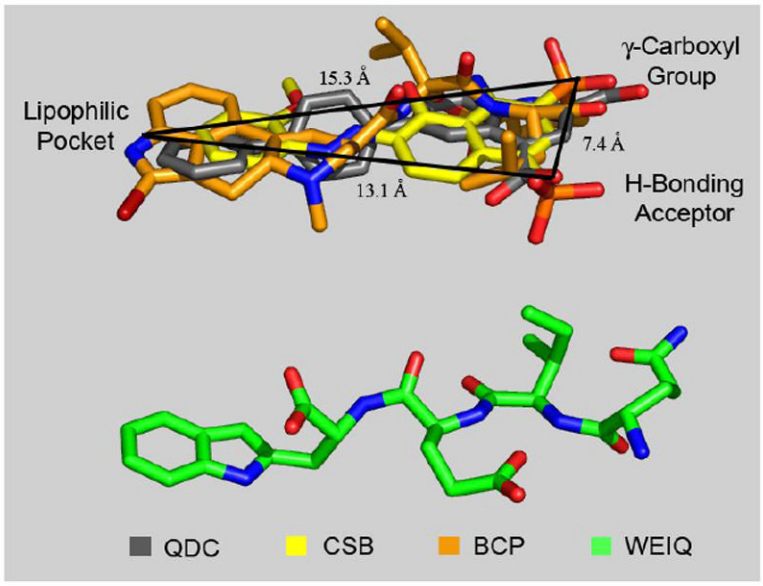
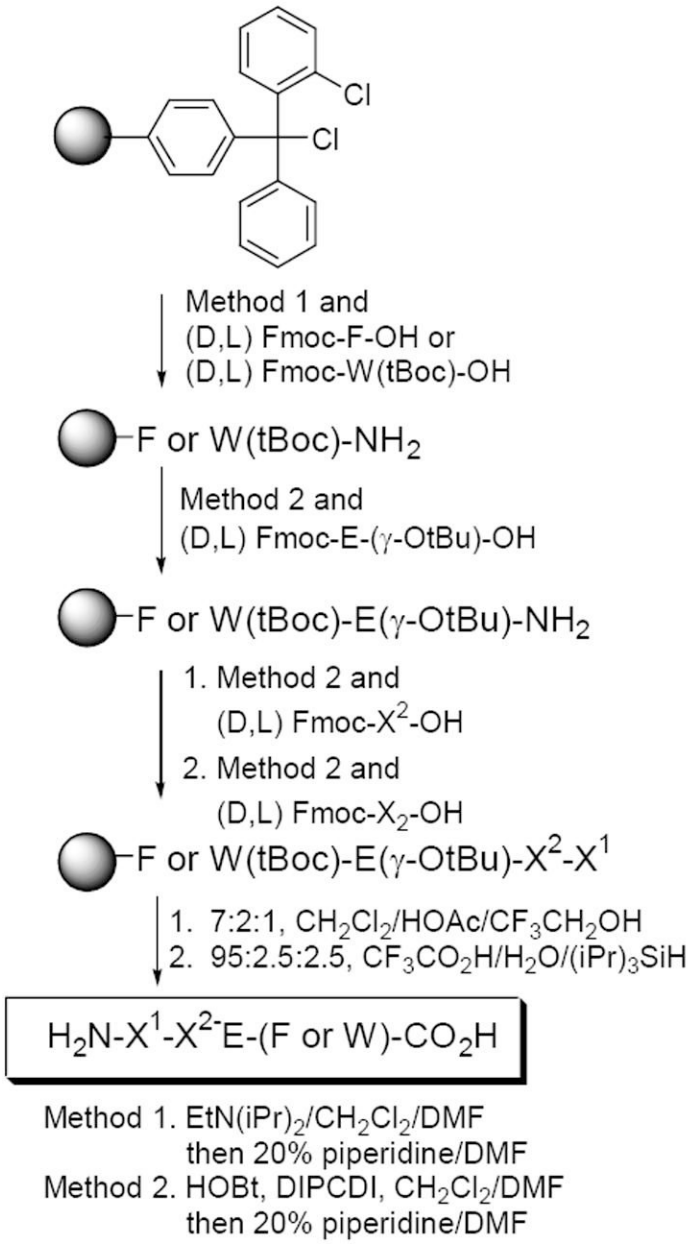



**Fig 5.**

An extended conformation of the tetrapeptide $_{L}Q_{D}I_{L}E_{D}W$ (green) aligned in relation to a computationally-derived VGLUT superposition pharmacophore model[1] composed with QDC (grey), one half of CSB (yellow) and BCP (orange), in which the ligands are correlated together with defined common pharmacophore comparison points (γ-carboxyl group, H-bonding acceptor and lipophilic pocket). Inter-atomic distances noted.




**Scheme 1.**
Synthesis of target tetrapeptides containing a glutamate at position 3.

**Table 1**

Inhibition of VGLUT by Tetrapeptides[1]

| X[1] | X[2] | X[3] | X[4] | $^3$H-L-Glu uptake (% of control)[2] |
|---|---|---|---|---|
| ***Library 1*** | | | | |
| AA[3] | AA | E | W | |
| Q | AA | E | W | 56 ± 1% |
| Q | W | E | W | 66 ± 4% |
| Q | I | E | W | 38 ± 5% |
| D-Q | D-I | L -E | D-W | 35 ± 3% |
| L-Q | D-I | L-E | D-W | 28 ± 3% |
| ***Library 2*** | | | | |
| AA | AA | E | F | |
| N | AA | E | F | 63 ±17% |
| W | AA | E | F | 36 ± 2% |
| W | N | E | F | 13 ± 3% |
| D-W | L-N | D-E | D-F | 41 ± 1% |
| ***Other*** | | | | |
| Congo Red (2 μM) | | | | 31 ± 2% |

[1] Tetrapeptides tested as racemic mixtures at 2mM.

[2] Control rate for $^3$H-L-glutamate uptake was 1847±130(n=17) pmol/min/mg protein.

[3] AA = 19 different amino acids.